CHROM. 4282

A sensitive method of documentation of porphyrin and hemin thin-layer chromatograms

In thin-layer chromatography of porphyrin esters[1-5] there is a real need for a method which permits, by a special but simple technique, the preparation of permanent records of the porphyrins on the chromatogram, which are excited to red fluorescence by long-wave UV light. The documentation should also be of high sensitivity and permit the simultaneous recording of the porphyrins and hemin as a complementary procedure to the simultaneous separation of these cyclic tetrapyrroles as methyl esters on silica gel thin-layer plates[3,4]. By using a camera with a normal lens plus specific filters and powerful UV lamps arranged in a special apparatus we obtained good reproducible photographs in black and white as well as slides in color. Light values for different films are presented. The method can be conveniently used to estimate the composition of a porphyrin mixture separated on a thin-layer plate or card, independent of viewing it directly under UV radiation.

Experimental

Thin-layer chromatography of the porphyrins, as their methyl esters, was carried out on silica gel plates (as described for Silica Gel H[1]) and several other precoated plates or cards[2], *e.g.* Silica Gel F_{254} plates (Merck, Darmstadt, Germany), Silica Gel plates DSF-A (Camag, Muttenz, Switzerland), and silica gel aluminum cards (Riedel-de Häen, Seelze-Hannover, Germany). The standard solvent system in analytical work for the separation of porphyrin esters according to their number of carboxylic acid methyl ester groups consists of benzene–ethyl acetate–methanol (85:13.5:1.5)[3]. Before separating the porphyrin esters in this solvent system a short run once or twice in a chloroform–methanol (130:20) solvent mixture is recommended for the formation of a narrow starting line[4]. The details of the chromatographic methods have been described earlier[1-4] and are presented fully elsewhere[5].

The porphyrins used were isolated from cell suspensions of *Achromobacter metalcaligenes*[6] on Silica Gel F_{254} plates and identified by their absorption spectra in chloroform[2,5]. Protohemin, obtained from Fluka (Buchs, Switzerland), was esterified, then purified by TLC[1] and analyzed by spectrophotometry[3].

The setup for photographing the porphyrins on silica gel thin-layer chromatograms is sketched in Fig. 1. By means of the data given in the legend to Fig. 1 the setup is easy to construct. Primarily it consists of two Desaga UV lamps 13 1000 (366 nm) (Desaga, Heidelberg, Germany), a camera and lens plus specific filters, a camera bracket and a base plate. The cameras and objectives used in this study are: an Exacta Varex IIa® (Ihagee Kamerawerk, Dresden, Germany) with a Jena T lens (1:2.8; f = 50 mm); and a Zeiss Ikon Contaflex (Zeiss Ikon, Stuttgart, Germany) with a Zeiss Tessar lens (1:2.8, f = 50 mm) and an additional A28 lens (f = 1 m). The room must be completely darkened for the photographic recording.

The filters found empirically suitable both for black and white and color films were studied for their efficiency for the photographic documentation of porphyrins by spectrophotometry as shown in Fig. 2. The following B + W filters (Johannes Weber KG, Wiesbaden, Germany) are employed for the photographic recording of the

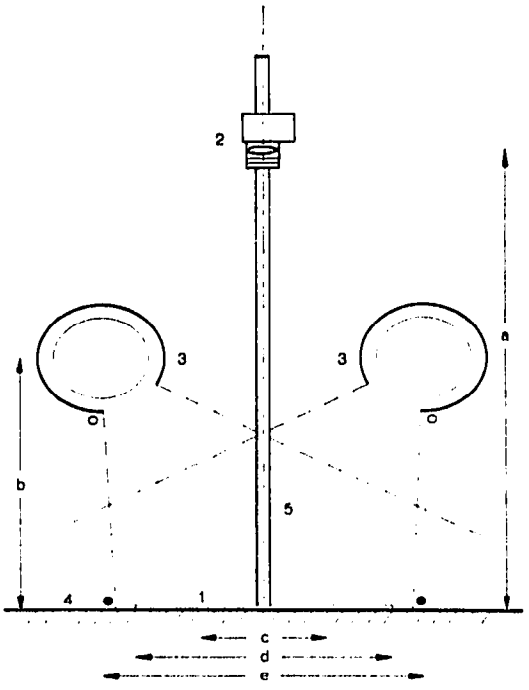

Fig. 1. Front view of the setup for fluorescence photography of porphyrins on silica gel thin-layer chromatograms (1). The other parts are: A single lens mirror reflex camera with a normal photographic objective (2), the filters are given in Table I; two Desaga UV lamps 131000 (366 nm) (3); a mat-black base plate (4); and a stand for vertical adjustment of the camera holder. The distance from the lens to the chromatogram is 55 cm (a) for plates 20·20 cm (d), and 40 cm for plates (cards) 10·20 cm (c). This variation in the distance has no influence on the light values given in Table I. The distance of the UV lamps (3) from the base plate is 23 cm (b), and from the optical axis 12.5 cm (e/2). The UV lamps are placed in such a way that the lower edge of each lamp (○) is projected on the black base plate 1 cm beyond the edge of a silica gel plate 20·20 cm (●). For photographic data (filter pack, light values and exposure times) see Table I.

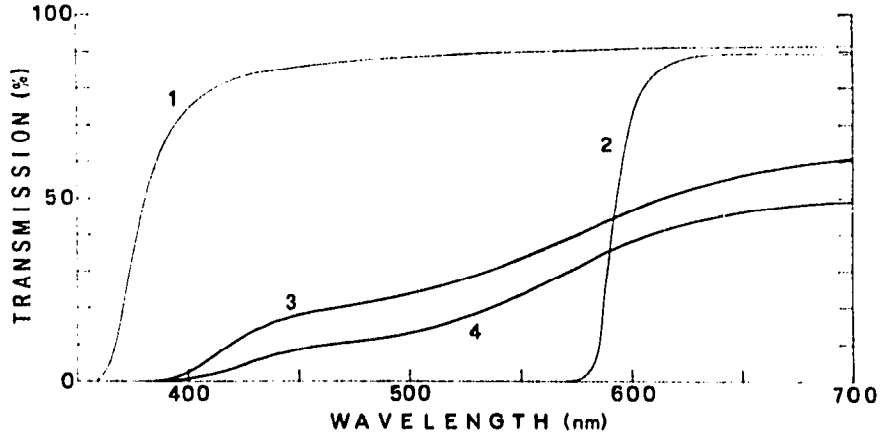

Fig. 2. Optical density in the visible range of the filters used for porphyrin photography in silica gel thin-layers. The filters were measured in a double beam Hitachi–Perkin Elmer EPS-3T spectrophotometer. 1 = 010; 2 = 090; 3 = 010 + KR 12; 4 = 010 + KR 12 + KR 6. For the origin and description of the filters see text.

fluorescence of porphyrins on thin-layer chromatograms: 010 = UV-Haze; 090 = light red; KR 12 and KR 6 for color correction against bluish tinge.

The photographic materials used in this laboratory are as follows:

Black and white negative: Ilford FP4 (ASA 125, DIN 22), developed in Ciba-Ilford Microphen; Agfa Isopan ISS21 (ASA 100).

Black and white reversal: Agfa Dia-Direct (ASA 32, DIN 16).

Color negative: Agfa CN S (ASA 80, DIN 20).

Color reversal: Agfacolor CT 18 (ASA 50), Kodachrome II (ASA 25, DIN 15), Kodachrome X (ASA 64, DIN 19).

The photographic detection of the red-fluorescent porphyrins on thin-layer chromatograms is achieved by using the arrangement shown in Fig. 1 and the conditions described in its legend in conjunction with the filters and light values listed in Table I.

TABLE I

LIGHT VALUES AND FILTERS FOR PHOTOGRAPHY OF PORPHYRIN–SILICA GEL THIN-LAYER CHROMATOGRAMS IN COMBINATION WITH THE SETUP SHOWN IN FIG. 1

Film	*Light value at lens aperture f/5.6 Exposure index 100 ASA (21 DIN)*	*Exposure time (min)*	*Filters*
Black and white negative	— 1	1	
	— 5[a]	16	090
Black and white reversal	— 3.5	6	090
Color negative	— 2	2	010 + KR 12 + KR 6
Color reversal	— 2	2	010 + KR 12
	— 2.5	3	010 + KR 12 + KR 6

[a] This value has to be used for the simultaneous recording of hemin.

Results and discussion

A method is described for the photographic recording of porphyrins on chromatograms under long-wave UV light. The data given in Table I were obtained using the following silica gel preparations: Silica Gel H, pre-coated F_{254}, and DSF-A plates and DC aluminum cards SI (see *Experimental*). The light values in Table I, ascertained with new UV lamps, have a range of ± 0.5 and will be reduced by using older radiation sources. The data depend on the properties of the UV lamps and are only valid for the radiation of the Desaga 13 1000 lamp.

For black and white photographs, the light red filter (090) is the most favorable, as it has a transmission of 89% in the region of the emission maxima[7] of the porphyrin methyl esters between 625 and 635 nm (Fig. 2). Since no transmission is observed with the 090 filter for light of wavelengths below 600 nm, as shown by spectrophotometry (Fig. 2), the additional use of the UV filter is not necessary. Other filters which are useful for recording the fluorescence of porphyrin thin-layer chromatograms are the dark yellow, yellow-orange, orange-red, and dark red B + W filters. However, the dark red filter 091[8] does not show an equal sensitivity for the fluor-

escent light emitted by the different porphyrin esters; the transmission at 620 nm is 11%, and 55% at 635 nm.

For color films, the filter packs given in Table I are especially suitable for the original true recording of fluorescent porphyrins. With the filter combination, 010 + KR 12 + KR 6 (resp. 010 + KR 12), light of wavelengths below 600 nm was absorbed preponderately allowing a high sensitivity in the detection of the porphyrins, and resulting in dark red spots with a blue background on the slides in spite of long exposure times (Table I). The color reproduction of the Kodachrome II slides is more natural if correction filters KR 12 + KR 6 are used. However, for other color reversal films, such as Agfacolor CT 18, the KR 12 filter will do.

Use of the red 090 filter for black and white exposures results in the sensitivity of the recording exceeding that when the chromatogram is viewed by the human eye in the detection of very low concentrations of porphyrin methyl esters on silica gel layers under the powerful UV lamps arranged as in Fig. 1. The minimum amount of porphyrin ester recorded by photography is 0.001 μg. Fig. 3, which shows a chromatogram with increasing amounts of coproporphyrin, indicates that the intensity of fluorescence is dependent on the topical distribution of the substance in the thin-layer. The photogram represents a summation reproduced from three films at different chromatographic times: (a) after application; (b) after formation of a narrow starting line; and (c) after running. The spots exhibit the greatest intensity of fluorescence in position b.

Photography of porphyrin thin-layer chromatogram without filters[1] required a

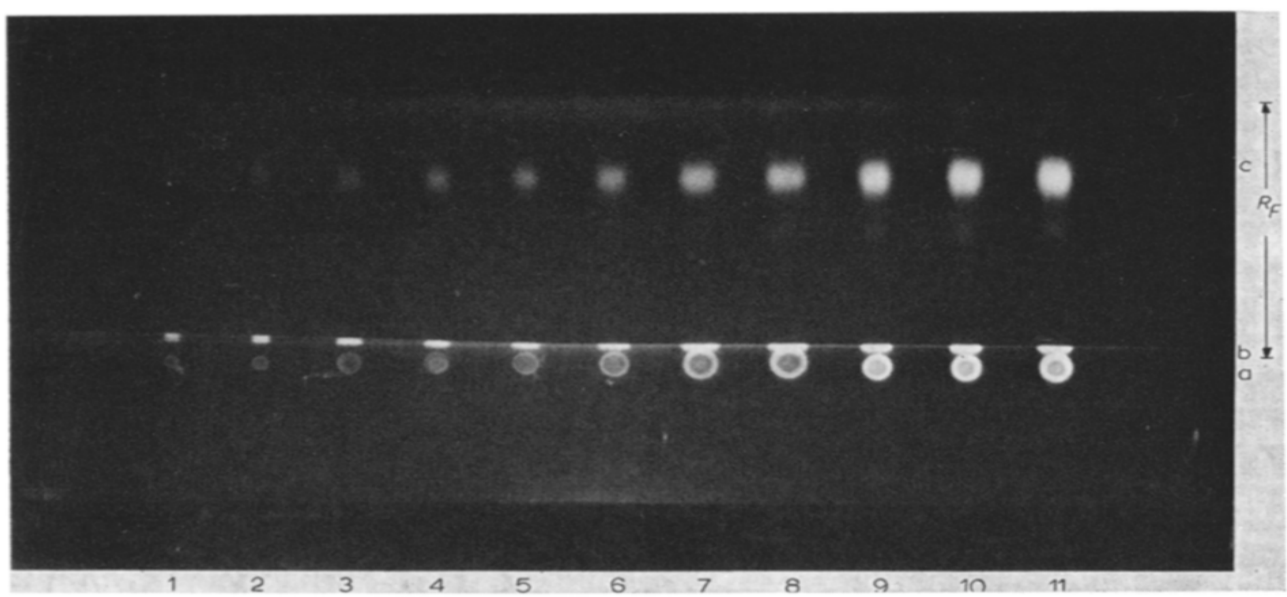

Fig. 3. Summation photogram of increasing amounts of coproporphyrin III tetramethyl ester on a Silica Gel F_{254} plate, which was photographed (film Ilford FP4, aperture $f/5.6$, shutter speed 40 sec) in three chromatographic situations: (a) after application of the coproporphyrin ester in chloroform solution by self-filling micro-pipettes; (b) after a two-fold short development of the plate in chloroform–methanol (130:20) for the formation of a narrow starting line; (c) after running in a fresh solvent mixture of benzene–ethyl acetate–methanol (85:13.5:1.5). For the production of this photograph, the films from situations a, b and c were pasted upon one another. The amounts in μg of coproporphyrin applied are: 1 = 0.003; 2 = 0.007; 3 = 0.014; 4 = 0.022; 5 = 0.029; 6 = 0.043; 7 = 0.072; 8 = 0.100; 9 = 0.207; 10 = 0.311; 11 = 0.414.

relatively high concentration of about 0.2 $\mu g/cm^2$, and resulted in a record in which the fluorescent spots show black against the white background of the plate[1,9], analogous to the record in Fig. 4c.

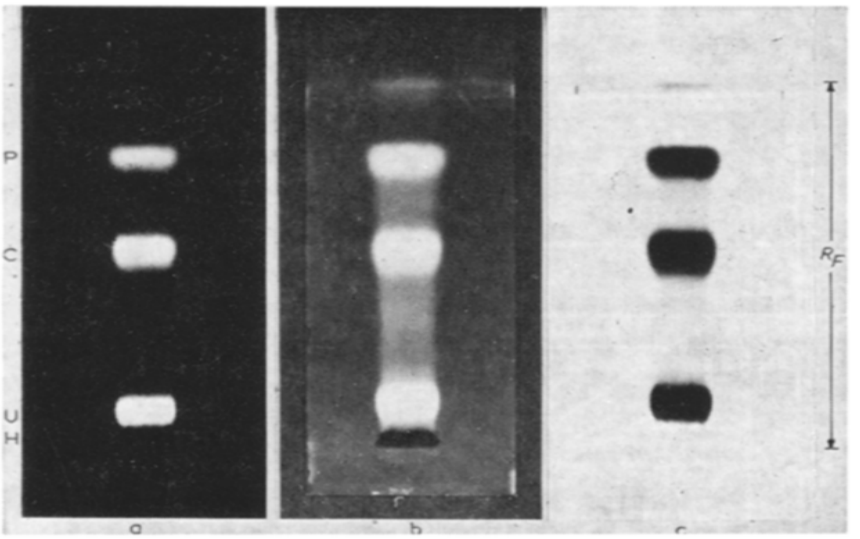

Fig. 4. Silica Gel F_{254} thin-layer chromatogram with proto-, copro-, uro-porphyrin and hemin methyl ester (P, C, U and H), developed in the solvent systems described in the legend of Fig. 3, but in a saturated atmosphere. The chromatogram was photographed with a black and white negative film for the documentation of porphyrins only (a) and for the additional detection of hemin (b). Recording of porphyrins as dark spots on white background was achieved with a black and white reversal film. Using filter 090, the film, lens aperture and exposure time are: a (Ilford FP4, *f*/5.6, 30 sec), b (Ilford FP4, *f*/4, 3 min), c (Agfa Dia-Direct, *f*/4, 9 min). Color photography of the same chromatogram with Agfacolor CT 18 was carried out under the following conditions: filter pack 010 + KR 12, aperture *f*/5.6, and exposure time 2 min.

Fig. 4 shows three different photographs of the same porphyrin and hemin chromatogram. The actual photographic conditions are given in the legend. The reproduction under the conditions of Fig. 4b permits the simultaneous record of hemin, which can be seen well in daylight as a brownish-black spot on the plate. Fig. 5 is the reproduction of a chromatogram of porphyrins from a bacterial culture (*Achromobacter metalcaligenes*), recorded using both a black and white negative (A) and a reversal film (B). Differences in the concentrations of the porphyrins separated on the chromatogram of about 1:400 (Fig. 5) could be detected by taking photographs under UV radiation with the technique reported here. The growth and incubation conditions of the bacterial system and the preparation of porphyrin methyl esters from it have been described in previous paper[6].

When photographing a series of porphyrin chromatograms we prefer records according to the Figs. 3, 4a and 5A, which give a natural impression of the fluorescence as well as possible in black and white. As shown in Figs. 3 and 5 the photographs allow an approximate estimation of the relative concentrations present in the porphyrin zones on the chromatograms.

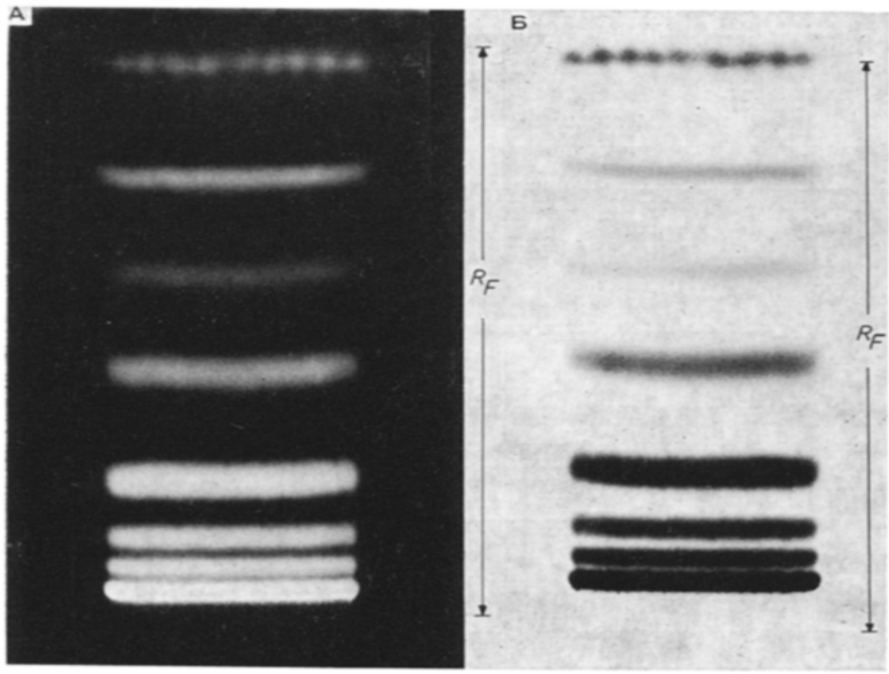

Fig. 5. Thin-layer chromatogram of porphyrin methyl esters (P-ME) prepared from cultures of *Achromobacter metalcaligenes* grown in a special medium with added δ-aminolevulinic acid[6]. According to Figs. 4a and c the photographic data were as follows: A, Ilford FP_4, *f*/5.6, 30 sec; B, Agfa Dia-Direct, *f*/4, 6 min. The porphyrin stripes from the start up to the front are: octa-, hepta-, hexa-, penta-, tetra-, tri- and dicarboxylic(proto) P-ME. The spots at the solvent front are lipids[2,4]. Results from a spectrophotometric analysis showed the difference in the concentration between tricarboxylic and uro P-ME to be about 1:400.

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